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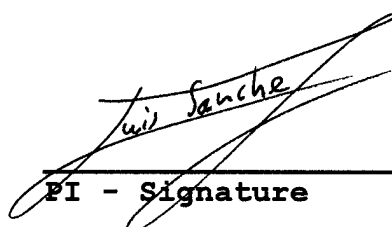
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## Introduction

### Subject:

Zn- $\alpha_2$ -glycoprotein (ZAG) is a soluble polypeptide of 41 kDa whose name derives from its tendency to be precipitated by zinc ions, its electrophoretical mobility in the  $\alpha_2$  region, and its carbohydrate content. ZAG is present in most bodily fluids, including serum, sweat, saliva, seminal plasma, cerebrospinal fluid, and urine (1). In addition, ZAG is present at high concentrations in about 40% of breast carcinomas, as well as in fluids from breast cysts (2, 3, 4, 5). ZAG is also induced by glucocorticoids and androgens in breast cancer cell lines (6). Taken together, these findings suggest that this protein participates in the development of mammary diseases including breast carcinomas.

Amino acid sequence analysis revealed that ZAG is surprisingly similar to class I major histocompatibility complex (MHC) proteins (7). Class I MHC molecules are cell surface proteins that present cellular peptides to cytotoxic T cells during immune surveillance (8). Each class I MHC protein consists of a membrane-spanning heavy chain associated with a soluble light chain called  $\beta_2$ -microglobulin. Crystal structure analyses reveal that the extracellular portion of the heavy chains fold into a shape ideally suited for peptide binding, including a characteristic groove located between two  $\alpha$ -helices that span an 8-stranded  $\beta$ -pleated sheet (9). The amino acid sequence of ZAG shares 30-40% identity with the extracellular portion of the class I MHC heavy chains, therefore ZAG is expected to adopt a similar three dimensional structure. Yet, our results indicate that ZAG is functionally different since it does not bind peptides or  $\beta_2$ -microglobulin (10). The sequencing of ZAG's gene confirmed the structural similarity and functional divergence (11). The coding sequence of the ZAG gene lacks the information for transmembrane or cytoplasmic segments, but otherwise is similar to those of class I genes. By contrast, the non-coding regions of ZAG and MHC genes differ widely in size and sequence. The gene of ZAG lacks the regulatory consensus sequence and interferon consensus sequence that are conserved in typical class I MHC genes. In addition, the gene encoding ZAG also differs of typical class I genes in its lack of polymorphism and in its location outside of MHC. These data indicate that ZAG is a soluble class I MHC-like protein that has diverged to develop a different function.

The function of ZAG in physiological and cancerous conditions has remained a mystery until very recently. However, studies from an unexpected direction have begun to clarify it. Approximately half of the patients with advanced cancer suffer cachexia, a massive wasting due to the depletion of the protein and fat stores of the body. Cachexia is a bad prognostic factor and it is believed to be responsible for the death of 20% of cancer patients [Argilés, 1997 #2; Stehle, 1997 #3; Tisdale, 1997 #860]. Tisdale and collaborators have characterized a lipid mobilizing factor from the urine of cachectic patients and identified it as ZAG. Additional experiments have shown that ZAG is overexpressed in cancers of cachectic patients, but not in cancers of non-cachectic patients (12). *In vitro* and *in vivo* assays revealed that ZAG can induce fat catabolism in adipocytes and reduce body mass in mice (13). These data suggest that ZAG is the factor responsible for body fat loss in cachexia. They also suggest that ZAG could regulate fat metabolism in breast tissue and other physiological or pathological conditions.

#### Purpose:

- To determine the three-dimensional structure of ZAG.
- To use the knowledge of ZAG's structure for understanding the function of ZAG and its relationship with classical MHC molecules.

#### Previous work:

The results of my previous work are in (10). Briefly...

**Purification of ZAG:** Structural studies require milligram amounts of pure protein. Therefore, I used fluid from breast cysts as a rich source of ZAG and developed a protocol for the isolation of ZAG. I purified the sample by hydrophobic chromatography, then by immobilized nickel affinity chromatography and finally by size exclusion chromatography. This procedure yielded 30 mg of ZAG per 100 ml of fluid.

**Analysis of the interaction between ZAG and  $\beta$ 2-microglobulin:** The heavy chains of all characterized MHC class I proteins bind  $\beta$ 2-microglobulin with high affinity. However, purified preparations of ZAG show a single band of 41 kDa on an SDS PAGE gel, with no sign of a 12 kDa  $\beta$ 2-microglobulin light chain. In order to ascertain if ZAG binds  $\beta$ 2-microglobulin, I incubated mixtures of both proteins in different conditions, and tried to detect the possible ZAG- $\beta$ 2-microglobulin complexes by several methods. These included gel filtration chromatography, ELISA, cross-linking and immunoprecipitation, but no complexes of  $\beta$ 2-microglobulin with ZAG were detected. Next, I used anti-ZAG and anti- $\beta$ 2-microglobulin antibodies to immunoprecipitate proteins from serum. The results indicated that serum contains free ZAG and free  $\beta$ 2-microglobulin, but no appreciable amounts of ZAG associated with  $\beta$ 2-microglobulin. Thus, ZAG does not bind  $\beta$ 2-microglobulin.

**Analysis of the possible peptides bound to ZAG:** I tested acid eluates from purified ZAG and a purified class I MHC molecule (H-2K<sup>d</sup>) for the presence of bound peptides. N-terminal sequencing revealed the presence of peptides in the K<sup>d</sup> acid eluate, but no peptides in the ZAG eluate, demonstrating that ZAG does not bind peptides.

**Thermal stability of ZAG:** MHC class I heavy chains are unstable in the absence of peptide or the  $\beta$ 2-microglobulin light chain (14), whereas ZAG exists as a class I heavy chain-like monomer without bound peptides. In order to compare the thermal stability of ZAG with a class I molecule, I obtained a melting curve for ZAG by recording the circular dichroism signal at 223 nm as a function of increasing temperature. Melting curves are characterized by their temperature at the transition midpoint or  $T_m$ , with a higher  $T_m$  indicating a more stable protein. ZAG denatures with a  $T_m$  of 65°C, as compared to 57°C for peptide-filled H-2K<sup>d</sup> and 45°C for empty K<sup>d</sup> (15). Thus, unlike conventional class I MHC molecules, ZAG does not require a light chain or bound peptide to fold into a stable structure.

Production of monoclonal antibodies against ZAG: The availability of pure ZAG allowed the production of monoclonal antibodies. I characterized nine monoclonal antibodies that react to ZAG, which were used in co-crystallization attempts with ZAG and for immunoaffinity purification of ZAG.

Crystallization of ZAG: I made numerous crystallization experiments with the pure protein, but I did not obtain crystals, even after repeated trials. Therefore, I subjected ZAG to different treatments, in order to control the potential variables that could be inhibiting its crystallization. These experiments included partial and total deglycosylation of ZAG, controlled proteolytic digestions, removal of potential ligands and co-crystallization of ZAG with fragments of different monoclonal antibodies. Eventually, I was able to obtain crystals of desialiated ZAG, but the crystals were not adequate for x-ray diffraction experiments. Additional analysis using non-denaturing gels demonstrated that ZAG purified from breast cyst fluid was less homogeneous than plasma ZAG. I therefore purified ZAG from commercially available human plasma using immunoaffinity chromatography followed by anion exchange and hydrophobic chromatography. This strategy yielded 8 mg of ZAG per liter of plasma, which allows an ample protein supply for crystallization trials. Using a sparse matrix factorial screening method (16), I obtained well ordered crystals in 30% PEG 4000, 0.1 M sodium acetate pH 4.6, 0.2 M ammonium acetate.

Data collection from ZAG crystals: ZAG crystallizes in space group  $p2_12_12$  with unit cell dimensions of 105 Å x 132 Å x 118 Å. The crystals diffracted to ~ 5 Å resolution at room temperature using an RAXIS image plate detector mounted on a rotating anode generator. The radiation sensitivity of ZAG crystals made it impossible to collect high resolution data at room temperature. However, by transferring protein crystals to a proper cryoprotectant, the crystals can be flash-cooled under a stream of nitrogen gas at -165°C. At this temperature, radiation decay is virtually eliminated (17) and I could use the high intensity of synchrotron radiation to collect high resolution reflections. Using this method, the effective resolution limit of the ZAG crystals extended to ~3.2 Å at Cornell High Energy Synchrotron Source (CHESS).

## Body

### Results:

The determination of the three-dimensional structure of a protein by x-ray crystallography is a process accomplished in several steps. The first step is the isolation of milligram amounts of protein that is pure, both chemically and conformationally. The second step is the production of crystals of the purified protein that diffract to atomic or near atomic resolution. The third step is the analysis of crystals under x-rays. These steps were already accomplished at the beginning of this project. However, data obtained by x-ray analysis are not enough to solve the structure of macromolecules. Each datum has to be complemented by an additional value, its "phase", in order to reconstruct the three-dimensional image of the molecule. To obtain those phases, I followed two parallel strategies, molecular replacement (MR) and multiple isomorphous replacement (MIR).

MR is a purely computational approach that provides phase information by replacing the unknown structure by the structure of a related protein that is already known. The corresponding phases are biased towards the related protein, but they can be adequate as initial values to be refined. I tried molecular replacement with many models, including class I MHC, class II MHC, and class I homologs like FcRn, CD1, and HFE. I also tried diverse fragments and combinations of those models. However, these attempts remained unsuccessful for many months. Therefore, I concentrated my efforts in the alternate strategy, MIR.

MIR is an experimental approach where protein crystals are soaked with different compounds containing heavy atoms. The x-ray data sets of the soaked crystals are compared with the data set from a control crystal by Patterson analysis. In favorable conditions, the comparison will allow to calculate the phases. The search for the right soak can require many trials and the available time at synchrotron sources is limited. Therefore, I varied my crystallization conditions until I got crystals that diffract to higher resolution (2.9 Å) without requiring synchrotron sources of x-rays. Then, I analyzed hundreds of crystals soaked with different heavy atom compounds and collected approximately forty data sets in the search for the right compound. Eventually, the analysis of six data sets from crystals soaked with mercury acetate and platinum tetrachloride provided initial phase values.

The initial phase values were refined taking advantage of the constraints imposed by solvent flipping and non crystallographic symmetry. The resulting electron density map allowed the trace of the chain of ZAG in the four molecules that are present in the asymmetric unit. The model is still being subject to alternate cycles of model building with O, and refinement with X-PLOR. At present, the model includes all diffraction data between 20 and 2.9 Å and has an R<sub>free</sub> of 30.9% (R<sub>cryst</sub> 26.1%). It includes all the residues of ZAG, except the first five residues and the last three ones, which appear to be disordered. The model also includes ZAG's carbohydrate chains, except the most distal residues.

#### Discussion:

ZAG has a "L" shape that is very similar to the ones of the class I MHC heavy chains. Considering that the MHC heavy chains are stabilized by the packing of β2-microglobulin against the angle of the "L", it is surprising that ZAG can attain its remarkable stability without interacting with β2-microglobulin. Analysis of the structure shows that ZAG has a stabilizing hydrogen bond network between its first two domains and the third one, which are forming the two sides of the "L". The surface area buried in the interface between those domains is also bigger in ZAG (880 Å<sup>2</sup>) than in MHC molecules (500 Å<sup>2</sup>).

The most striking result of the crystallization analysis is the presence of additional non-peptidic electron density in the place homologous to the peptide binding groove of class I MHC molecules. This additional electron density is surrounded by aromatic residues from ZAG. These observations indicate that ZAG indeed carries a hydrophobic non-peptidic ligand. Therefore, the role of ZAG in lipid mobilization could be due to ZAG itself, or could be due to the ligand carried by ZAG. I am currently using gas chromatography and mass spectrometry to identify ZAG's ligand.



### Experimental methods:

Data collection: Cryoprotected crystals of ZAG were flash-cooled by dipping them in liquid nitrogen. The cooled crystals were mounted under a stream of nitrogen gas at -165°C. The x-ray diffraction pattern of the crystals was collected by using a Rigaku rotating anode x-ray generator and a RAXIS2 image plate detector. Data were processed and scaled with the HKL software package (18).

Phase determination: I tried to obtain the phases for each x-ray reflection by molecular replacement using AMoRE (19). This strategy was unsuccessful, therefore, I obtained the phases by molecular isomorphous replacement using the program Sharp (20) helped by programs of the software package CCP4 (21).

Phase refinement: The initial phase values were refined by solvent flipping using Solomon, from the CCP4 package. There are four molecules of ZAG in the asymmetric unit. So, after calculating the non crystallographic symmetry axes, the phases were further refined using DM, from the CCP4 package.

Model building and refinement: ZAG's sequence has been built in the electronic density of each molecule in the asymmetric unit using the program O (22). The model is being refined with X-PLOR (23).

### Statement of work:

Objective 1: Determination of the three dimensional structure of ZAG

Task 1: Months 1-8: Molecular replacement analysis of data from crystals of native ZAG.  
Completed.

Task 2: Months 1-8: Search for heavy atom derivatives of ZAG crystals.  
Completed.

Task 3: Months 9-15: Data collection of heavy atom derivatives of ZAG crystals.  
Completed.

Task 4: Months 16-24: Determination of ZAG crystal structure by combination of the results from task2 and tasks 2-3.

Task planned for next year.

Objective 2: Comparison of the structure of ZAG with the structures of MHC proteins

Task 5: Months 25-30:  
Task planned for the final year.

Objective 3: Analysis of the function of ZAG

Task 6: Months 9-21: Extraction, isolation and identification of the ligand or ligands of ZAG.

Task planned for next year.

Task 7-9:  
Tasks planned for the final year.

## Conclusions

The three-dimensional structure of ZAG is solved. The parameters are already acceptable, but I think that they can be improved further because the refinement is still in progress and because the crystals will diffract better at a synchrotron.

The three-dimensional structure reveals that ZAG carries a non-peptidic hydrophobic ligand.

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